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Abundance and distribution of anaerobic protozoa and their contribution to methane production in Lake Cadagno (Switzerland)

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1. SUMMARY

In the uppermost layers of the anoxic sediment in Lake Cadagno, 9 different species of anaerobic protozoa were identified. The total number of these organisms was about 580 cells · ml^{-1} sediment. Most of these protozoa contained endosymbiotic methanogenic bacteria which in total amounted to 10^6 methanogens · ml^{-1} sediment. In addition to the methanogenic endosymbionts, cells of *Metopus setosus* and *Caenomorpha lata* also contained a non-fluorescent bacterial rod inside the cytoplasm. In some individual cells of *C. lata* this second type of endosymbiotic bacterium was sometimes the only endosymbiont observed. Contrary to earlier suggestions, anaerobic protozoa do not seem to play a major role in methane production at least in Lake Cadagno. No significant methane production due to the anaerobic protozoa and their methanogenic endosymbionts was found in situ. Isolated ciliates and amoebae produced methane at 12°C, but not at 6°C, probably as a result of temperature limitation. In the sediment of Lake Cadagno sulfate reduction seemed to be the dominant terminal degradation process.

2. INTRODUCTION

Anaerobic protozoa occur either free-living in anoxic marine and freshwater habitats or as inhabitants of the gastrointestinal tract of other organisms [1–4]. Endosymbiotic methanogenic bacteria have hitherto been found only in free-living anaerobic protozoa and probably in termite flagellates [5–7]. Methane production as a result of a presumably physiological interaction was shown for ciliates and amoebae containing high numbers of methanogenic endosymbionts [5,6]. In anoxic environments, up to a few hundred of these protozoa per ml were found by several authors [1,8,9]. However, the extent to which anaerobic protozoa are involved in mineralization of organic matter and in methane production in anoxic environments is still not known [5,8]. To clarify this issue, it is necessary to determine the numbers of

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protozoa and endosymbionts as well as their rates of methane production. So far only one study exists on the occurrence of sediment strata in which the bulk of methane presumably originated from anaerobic ciliates [8].

The present paper reports on field studies of anaerobic protozoa, their abundance and distribution in the anoxic sediment of Lake Cadagno and their endosymbionts. In addition, we present data on rates of methane production in sediment samples and by isolated protozoa.

3. MATERIALS AND METHODS

3.1. Sampling site

Lake Cadagno is an alpine lake situated 1923 m above sea level in the Piora Valley in the south of Switzerland. The lake is about 850 m in length and 420 m wide, with a maximum depth of 21 m. During the entire year the lake is stratified and subdivided into a salt-rich anoxic monimolimnion and a salt-poor oxygenated mixolimnion (crenogenic meromixis; C. Del Don, personal communication).

3.2. Field sampling

All sediment samples were taken as sediment cores with Plexiglas tubes 80 cm in length and 9 cm in diameter. The two sampling sites marked by buoys were located at 21 m water depth in the middle of the lake (SP1) and at 12.5–13.5 m water depth near the southern shore (SP2). Subsamples were taken immediately after retrieval of the sediment cores by syringes, either directly from the surface or through lateral pores in the tubes. The pores were spaced 1 cm apart and were covered by adhesive tape. Subsamples were transfered into pregassed (N_2) glass flasks, and headspaces were flushed immediately with nitrogen gas. The samples were stored in a cold box in the dark until further processing. Subsamples for enumeration of protozoa were taken in increments of 1 cm, from 5 cm above the sediment to 5 cm into the sediment.

3.3. Analytical procedures

For determination of methane and sulfate concentrations in the lake, an in situ dialysis membrane pore water sampler (DPS) was used [10,24]. The DPS consisted of a plastic plate with 64 compartments, each at a distance of 1.9 cm and with a volume of 19 ml. The compartments were filled with distilled water and covered on both sides with cellulose dialysis membranes (Visking Seamless C-110, Union Carbide, Chicago, IL, U.S.A.). The DPS was vertically inserted half way into the sediment by a diver, the first compartment above the sediment was marked by tape. After incubation for 2 weeks, the DPS was retrieved and immediately covered with an isolation foil to prevent losses of volatile compounds. Pore water samples were taken by syringes through the dialysis membrane. For methane analysis, 2 ml of pore water were injected into 12-ml glass tubes containing 100 μl of 10% HNO_3 as fixative, and sealed with butyl rubber stoppers. After shaking and equilibration, the CH_4 concentration in the gas phase was measured by gas chromatography (GC R-1A, Shimadzu, Kyoto, Japan, with flame ionization detector operating at 200°C). 180 μl of the gas phase was injected by a sample loop on a MS5A-column (Supelco, Bellefonte, PA, U.S.A.) operating at 200°C with N_2 as carrier gas at a flow rate of 50 ml · min^{-1}. Sulfate was determined by ion chromatography (Sykam, Gilching, F.R.G.; equipped with a conductivity detector). One-ml pore water sample was added to 20 μl formaldehyde as fixative and diluted 20-fold for analysis. Twenty microliters of the diluted sample water were injected into an anion separation column (modified LCA-A01, Sykam, Gilching, F.R.G) with 4 mM $NaHCO_3$ and 0.5 mM Na_2CO_3 as eluent and a flow rate of 2 ml · min^{-1}. Oxygen, pH, temperature and conductivity were measured directly on the lake by using a combined electrode device (Hydropolytester, HPT-C, Züllig-AG, Rheineck, Switzerland).

3.4. Enumeration of protozoa

Living ciliates were enumerated in 0.5-ml replicate subsamples of the sediment cores by using electromigration [8]. To prevent poisoning of the samples by electrolytic gas production, separate electrode chambers were present. These chambers were connected to the migration chambers by agar bridge electrodes. They consisted of glass tubes

containing 10% Na_2SO_4 in 3% agar. For electromigration an electric field strength of 7.2 $V \cdot cm^{-1}$ was applied. Living amoebae were enumerated in homogeneous subsamples which were diluted 10-fold with filtered anoxic sample water. For counting, 0.5-ml plankton-counting-chambers (Hydro-Bios, Kiel-Holtenau, F.R.G.) were used, and each sample was counted 4 times.

3.5. Microscopy

Presumptive methanogenic bacteria were detected by using epifluorescence microscopy [11]. The number of endosymbiotic bacteria was determined either from photomicrographs as described earlier [6], or by means of a counting chamber (W. Schreck, Hofheim/Ts, F.R.G.). In the latter case, protozoa were washed in sterile sample water and defined number of cells were injected into 1 ml sterile water and disrupted by sonication, thereby liberating the bacteria. For photomicroscopy the protozoa were fixed in a mixture of 0.5% (v/v) formaldehyde and 0.3% (v/v) glutaraldehyde. Differential interference contrast optics (DIC) were used with a Zeiss or Leitz photomicroscope. Protozoa were identified according to Bick [12], Jankowski [2], Kahl [13] and Wetzel [1].

3.6. Methane production assays

Protozoa free of extraneous bacteria were obtained by washing. To do this, individual cells were isolated from sediment samples using micropipettes and incubated for 1 h in sterile sample water. Afterwards, defined numbers of cells were injected into 2 ml sterile sample water and incubated in 4.5-ml glass tubes sealed with butyl rubber stoppers. Sample water from the washing solution without protozoa was used as control. All steps were carried out in an anaerobic glovebox with a gas phase of N_2/H_2 (98%/2%, v/v). The tubes were incubated at 5 °C and 12 °C in the dark. Methane production was measured in headspace samples with a Perkin-Elmer Sigma 4B gas chromatograph (Perkin Elmer, Überlingen, F.R.G.) equipped with a Porapak R Column (1.8 m, 100/120 mesh) working at 80 °C and a flame ionization detector at 150 °C. After 48 h the experiment was stopped and the total number of living cells was determined by counting. Sediment incubation experiments were carried out with 30 ml homogeneous sediment material in 50-ml serum bottles sealed with butyl rubber stoppers and N_2 in the gas phase. The samples were incubated at 6 °C in the dark and methane was measured in the headspace. Sulfate was analyzed at the beginning and in the end of the experiment.

4. RESULTS

4.1. Lake Cadagno

A typical profile of oxygen, temperature and pH in Lake Cadagno, which was measured in August 1988, is shown in Fig. 1. The sediment was permanently anoxic with a temperature of about 5 °C. The oxic/anoxic transition zone was found

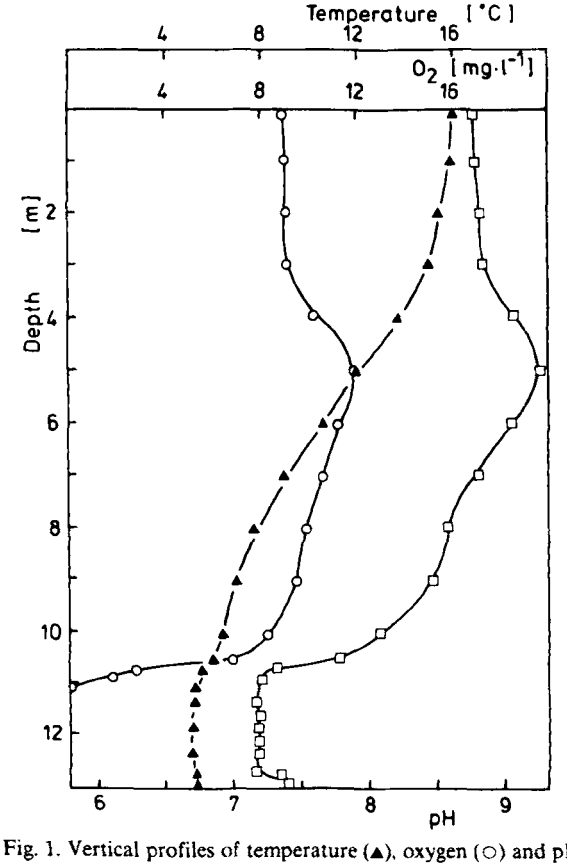

Fig. 1. Vertical profiles of temperature (▲), oxygen (○) and pH (□) in Lake Cadagno at sampling site 2 (SP2).

at 10–11 m depth, with an oxygen-free monimolimnion and an oxygenated mixolimnion. The slight increase in dissolved oxygen in the middle of the mixolimnion was probably due to photosynthesis of diatoms, dinoflagellates and green algae thriving in this water layer.

4.2. Abundance and distribution of anaerobic protozoa

The anoxic sediment of Lake Cadagno contained a great diversity of anaerobic microorganisms, including bacteria, amoebae, ciliates and flagellates. The sediment was covered by a thin (2–3 mm) red layer of purple sulfur bacteria, the majority of which were *Chromatium* sp. (S. Wagener and C. Del Don, unpublished results). These bacteria most likely originated from the layer of phototrophic bacteria at 10–11 m water depth. In addition to other bacteria, the purple sulfur bacteria were often detected in the food vacuoles of anaerobic protozoa. Most of the protozoa were found in the upper 2 cm of the sediment, with a different distribution at the two sampling sites (Fig. 2). Along with *Metopus setosus* (Fig. 3a), *Metopus es, Metopus acidiferus, Brachonella* sp., *Plagiopyla nasuta, Caenomorpha lata* (Fig. 4a), and *Lacrymaria* sp. were found, as well as the large amoeba *Pelomyxa palustris* and a small *Pelomyxa*-like cell type. In addition, large numbers of heterotrophic nanoflagellates were often observed feeding on *Chromatium* sp. The most abundant protozoa were *Metopus setosus* cells (251 ± 150 cells $\cdot$ ml^{-1};) at SP2, which occurred in the first 2 cm of sediment and cells of *C. lata* at SP1 (54 ± 15 cells $\cdot$ ml^{-1}). Cells of *Pelomyxa* sp. were found only in the sediment, with 109 ± 46 cells $\cdot$ ml^{-1} at SP1 and 125 ± 50 cells $\cdot$ ml^{-1} at SP2 (table 1). During our measurements between June and September 1988, no significant changes in the abundance and distribution of the anaerobic protozoa were observed. Among the hundreds of ciliates observed only one dividing cell was detected, a cell of *Brachonella* sp.

4.3. Endosymbiotic bacteria and methane production

Endosymbiotic putative methanogenic bacteria were found in all of the anaerobic protozoa mentioned above (Table 1) by using epifluorescence

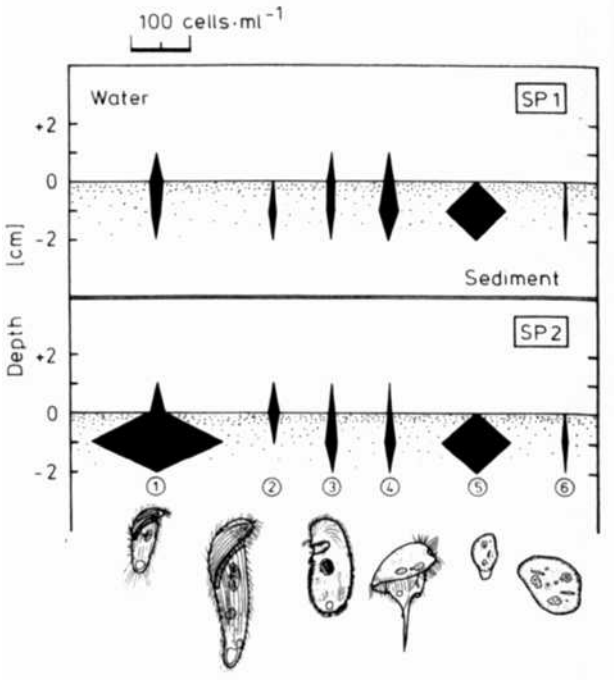

Fig. 2. Abundance and distribution of anaerobic protozoa in Lake Cadagno at 21 m (SP1) and 13 m (SP2) water depth; *Metopus setosus* (1), *Metopus es* (2), *Plagiopyla nasuta* (3), *Caenomorpha lata* (4), *Pelomyxa* sp. (5), *Pelomyxa palustris* (6). Pictures in part redrawn after Kahl [13].

microscopy. Next to fluorescent rod-shaped methanogens ($0.25\ \mu m \times 2.6$–$6.6\ \mu m$) another type of bacterium was observed in the cytoplasm of *Metopus setosus* and *Caenomorpha lata* (Figs. 3b, 4b). These latter, larger rods ($1\ \mu m \times 4$–$20\ \mu m$) exhibited no fluorescence and, therefore, were considered to be non-methanogenic endosymbionts. In all samples, individual cells of *C. lata* were sometimes observed to contain no detectable methanogenic bacteria, whereas the large non-methanogenic rods were still present in the cytoplasm of such cells (Fig. 5). The total numbers of endosymbionts in the cytoplasm of the most abundant anaerobic protozoa are listed in Table 2. The numbers of methanogenic bacteria were 6–10 times higher than those of the non-methanogenic rods. Multiplication of these numbers by the total number of protozoa in the sediment translated to 1.0–$1.3 \cdot 10^6$ endosymbiotic methanogens per ml sediment, considering only *M. setosus, C. lata* and the small *Pelomyxa*-like amoeba. Methane forma-

Table 1

Numbers of the most abundant protozoa above the sediment (0 to +1 cm) and in the sediment (0 to −2 cm) at sampling site 1 (SP1, 21 m water depth) and sampling site 2 (SP2, 12.5–13.5 m water depth)

	Cells · ml^{-1} sediment			
	SP1		SP2	
Sampling depth (cm):	0 to +1	0 to −2	0 to +1	0 to −2
Metopus setosus	35 ± 14	30 ± 11	45 ± 11	251 ± 150
Metopus es	n.f.	6 ± 2	n.f.	17 ± 6
Plagiopyla nasuta	11 ± 3	14 ± 5	11 ± 4	34 ± 8
Caenomorpha lata	16 ± 13	54 ± 15	3 ± 1	17 ± 7
Pelomyxa-like cell	n.f.	109 ± 46	n.f.	125 ± 50
Pelomyxa palustris	n.f.	3 ± 1	n.f.	3 ± 2
Ciliates ≤ 50 μm	–	26 ± 8	–	129 ± 55

Mean ± SD of 2–4 independent samples; n.f., not found.

Table 2

Numbers of endosymbiotic bacteria in the cytoplasm of anaerobic protozoa from Lake Cadagno

	Endosymbiotic bacteria (cell^{-1})	
	Fluorescent	Non-fluorescent
Metopus setosus [m]	490 ± 70	44 ± 10
Caenomorpha lata [m]	780 ± 90	124 ± 10
Pelomyxa-like cell [c]	9100 ± 900	n.f.

Mean ± SD of 5 cells using photomicrographs (m), and of 30 cells using counting chambers (c), n.f., not found.

tion by these anaerobic protozoa was tested with washed cells which were incubated at 6 °C and 12 °C. After 48 h, no significant methane was produced at 6 °C. In contrast, protozoa incubated

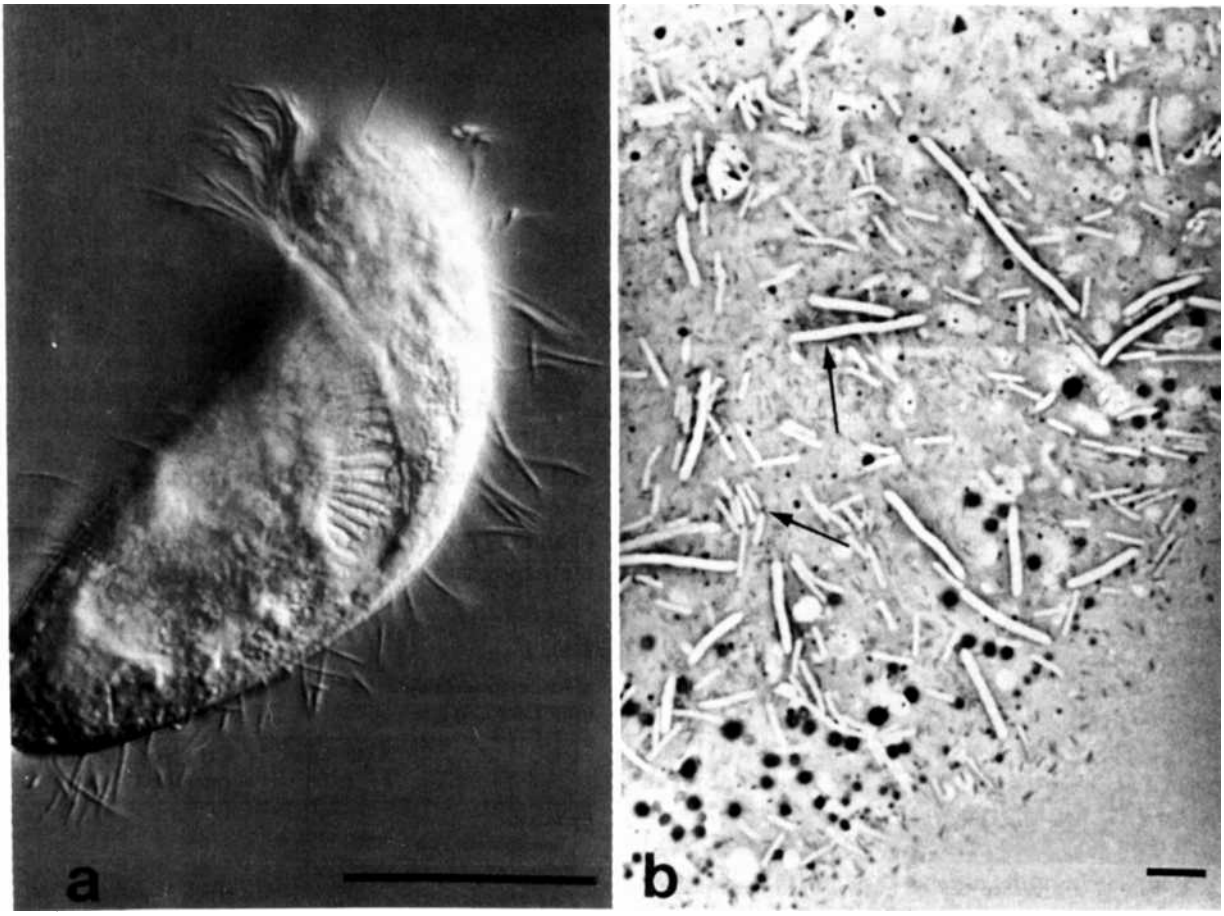

Fig. 3. *Metopus setosus*. (a) Fixed cell (DIC), bar represents 20 μm; (b) part of a squashed cell (phase contrast), showing 2 types of endosymbiotic bacteria in the cytoplasm (arrows). Bar represents 5 μm.

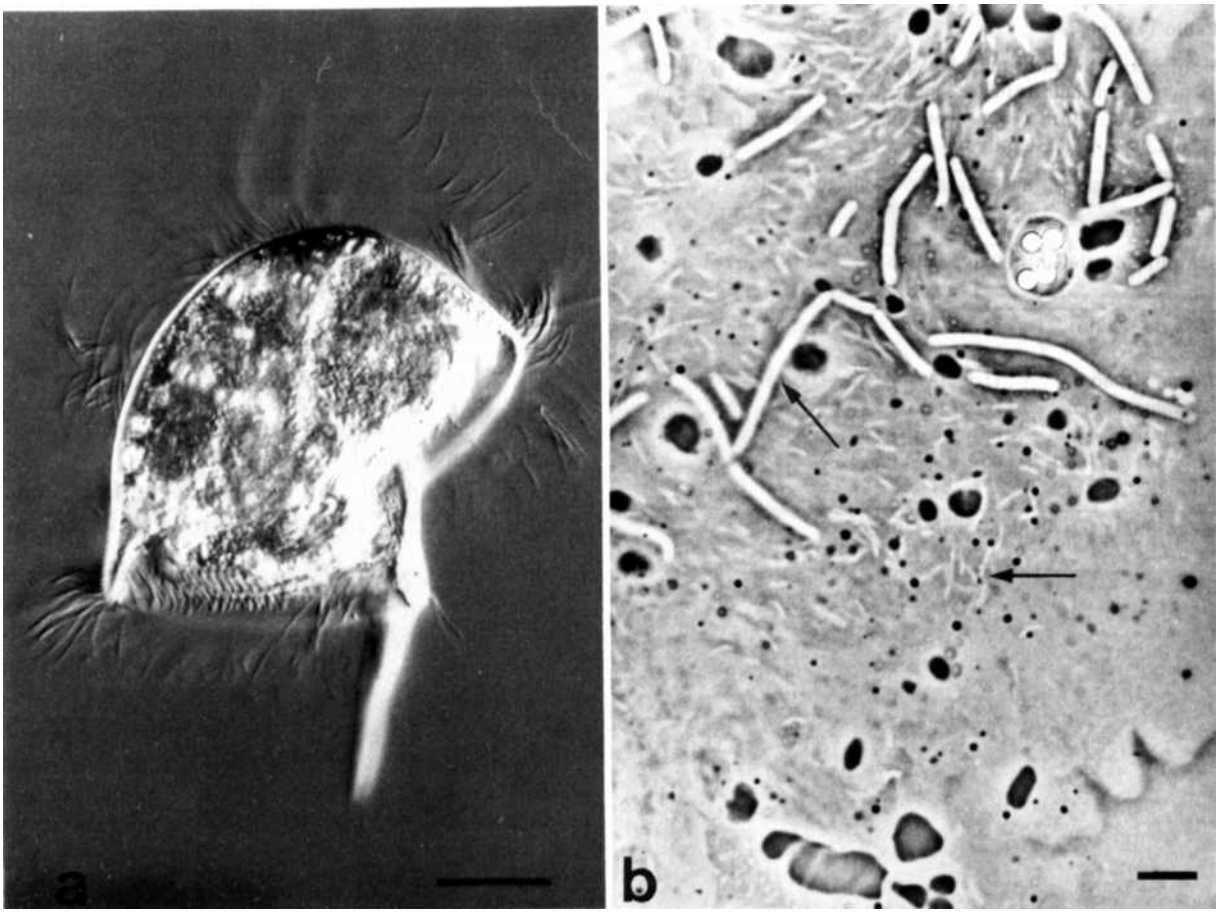

Fig. 4. *Caenomorpha lata*. (a) Fixed cell (DIC), bar represents 20 μm; (b) part of a squashed cell (phase contrast), showing 2 types of endosymbiotic bacteria in the cytoplasm (arrows). Bar represents 5 μm.

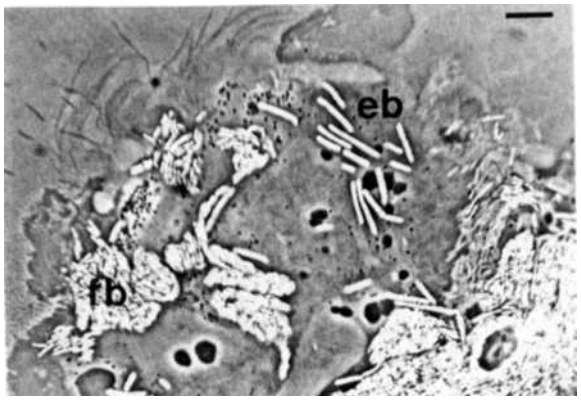

Fig. 5. Part of a squashed cell of *Caenomorpha lata* (phase contrast) with only 1 type of endosymbiotic bacteria (eb) in the cytoplasm and large vacuoles containing food bacteria (fb). Bar represents 5 μm.

at 12°C were able to produce methane. The rate of methane production was highest for *P. palustris* (Table 3) which contained the largest number of methanogenic bacteria (data not shown).

Table 3

Methane production at 12°C by different anaerobic protozoa from Lake Cadagno

	Cells · assay^{-1}	CH_4-production rate (pmol · cell^{-1} · day^{-1})
Protozoa		
Caenomorpha lata	120	0.9
Pelomyxa-like cell	340	6.7
Pelomyxa palustris	4	236.4
Control [a]		0.7

Mean incubation time: 48 h; [a] pmol · day^{-1}.

4.4. Methane and sulfate profiles

In the anoxic water column (monimolimnion) of Lake Cadagno high concentrations of sulfate were found. Above the sediment, the sulfate concentration was 2.9 mM at SP1 (21 m) and 2.5 mM at SP2 (12.5–13.5 m). In the uppermost 4 cm of the sediment at SP1, the sulfate concentration decreased down to 0.9 mM, whereas at SP2 the sulfate concentration increased to about 7 mM (Fig. 6), probably due to the influence of underwater springs nearby, which are under current investigation. At the two sampling sites the methane profiles were reciprocal to the sulfate profiles. At SP1 the methane concentration was about 60 μM above the sediment and increased continuously in the sediment, most probably due to diffusion from deeper sediment layers. At 1 m sediment depth, the methane concentration was 3.5 mM. At SP2 the highest concentrations of methane were measured above the sediment. The methane concentration decreased from 44 μM at 7.6 cm above the sediment to 11 μM at 4 cm in the sediment (Fig. 6). During our measurements at both sampling sites, no significant methane production was observed in the first 2 cm of the sediment where the abundance of anaerobic protozoa was highest.

To test for methane formation in these sediment layers we incubated the sludge at 6 °C (Fig. 7). During the first 2 days an increase of the

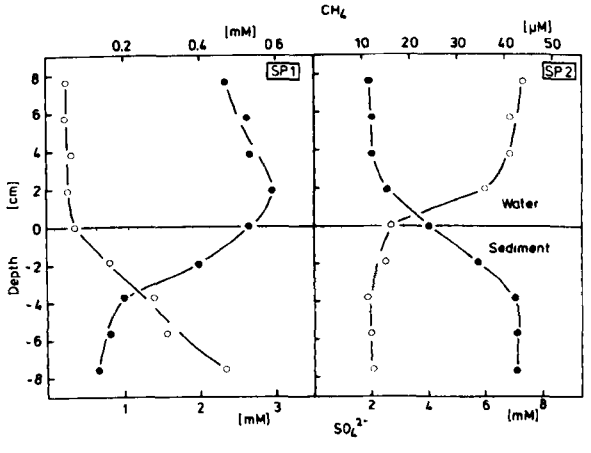

Fig. 6. Vertical profiles of sulfate (●) and methane (○) in Lake Cadagno at 21 m (SP1) and 13 m (SP2) water depth.

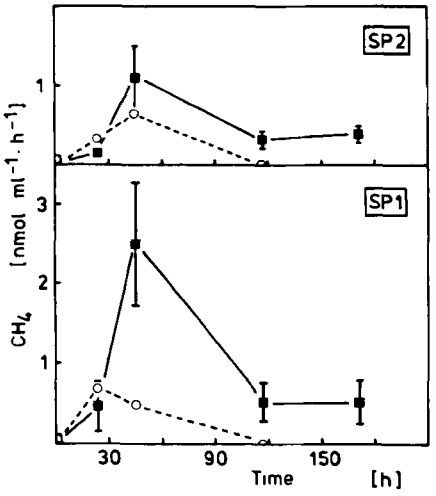

Fig. 7. Methane production rates of sediment samples from 21 m (SP1) and 13 m (SP2) water depth at 6 °C. Sediment material without (■) and with formaldehyde (○) as control.

methane production rate was observed. This effect was probably created by degassing sediment material even in the formaldehyde-treated samples and stimulation of methanogenesis due to mixing the sediment before incubation. After 48 h of incubation net methane production rates of about 48 and 10.5 nmol $CH_4 \cdot ml^{-1} \cdot day^{-1}$ were found. During the same time the sulfate concentration decreased to below 10 μM. After 5 days, a steady methane production rate of about 9 and 12.7 nmol $CH_4 \cdot ml^{-1} \cdot day^{-1}$ was measured.

5. DISCUSSION

5.1. Abundance of anaerobic protozoa

In the anoxic sediment of Lake Cadagno, different types of anaerobic protozoa were present. Ciliates and amoebae reached mean cell numbers of about 580 cells $\cdot ml^{-1}$ sediment. These results are comparable to recent reports: about 700 anaerobic protozoa $\cdot ml^{-1}$ were found in the sediment of a productive freshwater pond [9] and up to 650 anaerobic ciliates $\cdot ml^{-1}$ in a sludge backing pond [8]. Earlier studies also reported on the existence of a permanent, extensive population of free-living anaerobic protozoa in anoxic environments of fresh water systems [1,13]. Nevertheless,

the importance of such protozoa in the recycling of organic matter and in the anaerobic food chain is still unknown, despite the interesting presence of endosymbiotic methanogenic bacteria inside anaerobic protozoa [5].

5.2. Anaerobic food chain

In freshwater sediments, most of the anaerobic ciliates ingest bacteria including various types of phototrophic purple sulfur bacteria which are considered to be a food source. For example, the purple sulfur bacterium *Thiopedia* was found in food vacuoles of ciliates [9,14]. We observed the ingestion of *Chromatium* sp. by ciliates and flagellates. On the other hand, attempts to cultivate some anaerobic protozoa with various types of purple sulfur bacteria remained unsuccessful (C. Stumm, personal communication). These protozoa may require an additional food source apart from the phototrophic bacteria tested. In contrast, the anaerobic ciliate *Trimyema compressum* was able to grow with *Chromatium okenii* and other *Chromatium* sp. as sole food bacteria [15].

In aerobic environments where ciliates feed on phototrophic and heterotrophic nanoplankton, they themselves are a food source for e.g. rotifers. In the permanent anoxic sediments of Lake Cadagno, we detected the existence of a rather short food chain. Here, the anaerobic protozoa are the primary consumers of bacteria, and also the final consumers. There have been no reports of a predator of these protozoa in permanently anoxic, reduced environments.

5.3. Methane production in the sediment

The competition between methanogenic and sulfate-reducing bacteria for available substrates is well described for anoxic environments [16–18] and appears also to exist in Lake Cadagno. Profiles of methane concentrations showed a reciprocal correlation with the sulfate concentrations. If at all, methane production took place only rather deep in the sediment (site SP1) or slightly above the sediment (site SP2) where sulfate reduction was minimal, as concluded from the methane and sulfate profiles. Moreover, in sediment-slurry experiments, significant methane production rates were obtained only after sulfate concentrations had been reduced, indicating the existence of a competition between methanogenic and sulfate-reducing bacteria. Endosymbiotic methanogenic bacteria are probably not involved in this competition, since they should be able to obtain their substrates while inside the protozoan cell, and sulfate reducers were never reported as endosymbionts. Most of the anaerobic protozoa occurred in the upper 2 cm of the sediment where sulfate-reducing activity was dominant, as concluded from the sulfate profiles and sulfate reduction measurements (K. Hanselmann, manuscript in preparation). Methane production due to the anaerobic protozoa and their methanogens was therefore expected. Nevertheless, the previously reported speculation about the existence of sediment strata in which the bulk of methane is formed by anaerobic protozoa [5,6] could not be confirmed in our study. Although we found several hundreds of protozoa with about 10^6 endosymbiotic methanogens $\cdot$ ml^{-1} sediment, no significant methane production could be detected in situ. In addition, the incubation of isolated protozoa showed that methane formation is in fact measurable, provided the incubation temperature is raised from 6 to 12° C. These results indicate that methane formation from the endosymbiotic association between protozoa and methanogens in Lake Cadagno is temperature-limited. Temperature limitation of methanogenesis in general has often been reported [19–21]. Methane measurements with sediment material of Knaack Lake showed a methane production rate of 18.3 nmol $\cdot$ ml^{-1} $\cdot$ day^{-1} at 5.5° C [22]. In Lake Mendota the methane production rate varied between 5-320 nmol $\cdot$ ml^{-1} $\cdot$ day^{-1} at 4° C [19]. These rates are directly comparable to the rates we found for sediment of Lake Cadagno (9–48 nmol $\cdot$ ml^{-1} $\cdot$ day^{-1}). Unfortunately, there are no data available concerning anaerobic protozoa in these lakes. Methane production by anaerobic protozoa with their methanogenic endosymbionts was not measurable at 6° C (see Table 3), indicating that temperature has a significant impact on the metabolism of the host which, in turn, also influences the substrate availability for the endosymbiont.

Nevertheless, the methanogenic bacteria inside the protozoa occupy an interesting ecological

niche. They are protected against competition with sulfate reducers living in the same environment and if one assumes that sulfate reducers are a potential food source for the protozoa, these bacteria may end up as a carbon and energy source for the methanogens. In future studies, direct measurements of the total methanogenic activity, and that attributable to the anaerobic protozoa in situ would be desirable.

5.4. Counting of free-living methanogens

The number of methanogenic bacteria detected in anoxic sediments varies between 10^4–$10^5 \cdot ml^{-1}$ mud [16,19,23]. Enumeration was normally done by dilution techniques for which the mud had to be homogenized properly. Since protozoa are somehow fragile organisms, one is forced to wonder how many of the methanogenic bacteria counted originated from disrupted protozoa and how many were actually free-living. During homogenization of sludge, individual protozoa were sometimes disrupted, with release of endosymbiontic methanogens (S. Wagener, unpublished results). In future studies on the abundance of methanogenic bacteria in sediments, the existence of endosymbiotic methanogens should therefore to be taken into account.

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